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HEYDRICK, WACHTER and DWYER

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## HOST INFLUENCE ON THE CHARACTERISTICS OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS

FRED P. HEYDRICK, RALPH F. WACHTER and JAMES X. DWYER  
BIOLOGICAL SCIENCES LABORATORY  
FORT DETRICK, FREDERICK, MARYLAND

## INTRODUCTION

One objective of the virus research program at Fort Detrick is to demonstrate that physiochemical and biological properties of viruses can be altered by selection of, or modification of, the host system in which the virus is propagated, with the ultimate goal of obtaining viral agents whose biological characteristics can be predicted and/or controlled in the growth environment. An approach to this objective was to study the relationship of changes in lipid composition of Venezuelan equine encephalitis (VEE) virus that occur when the virus is propagated in different hosts and to determine if a correlation exists between the changes observed and the biological characteristics of the virus. Comprehensive analyses of the lipid of VEE virus propagated in a number of different hosts have been performed.

## MATERIALS AND METHODS

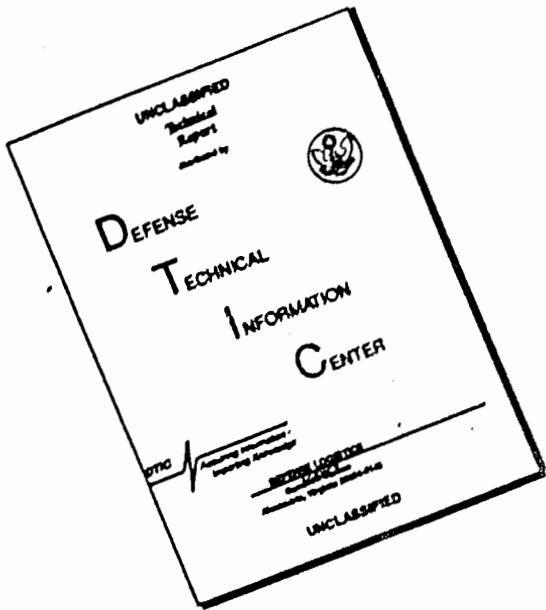
Hosts. The host materials used in this study were either embryos from 10-day eggs or chick fibroblast (CF) monolayers grown in Roux bottles containing lactalbumin hydrolysate medium with 10 per cent calf serum.

Virus. The VEE virus propagated in these tissues was the Trinidad strain which had received 13 passages in embryonated eggs. Unlabeled virus preparations were obtained from either chick embryos or CF monolayers and purified as described by Heydrick, Wachter and Hearn (1). Labeled virus was prepared by injecting 9-day eggs with 500  $\mu$ c  $P^{32}$  or by adding 10  $\mu$ c  $P^{32}$  per ml of maintenance medium prior to inoculation of the CF monolayers. At the end of a 20- to 24-hour period of label incorporation, the host systems were infected with unlabeled virus and virus preparations were obtained and treated as described above (1).

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419

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HEYDRICK, WACHTER and DWYER

Biological Characterization of Virus. Viral assays were performed by injecting 12- to 14-g mice by intracerebral (ic) and intraperitoneal (ip) routes with 10-fold dilutions of the preparation. Viral titers were expressed as mouse ic LD<sub>50</sub> and ip LD<sub>50</sub> per ml. The CF monolayer plaque technique for intact virus, as described by Colon and Idoine (2), was used to determine the virus plaque size.

Lipid Extraction and Analysis. Labeled or unlabeled purified virus was extracted at 50 C for 1 minute with 80 per cent ethanol and for two 15-minute periods with chloroform and methanol (2:1) in an atmosphere of nitrogen. Labeled or unlabeled homogenized embryos or pools of CF monolayers, after being washed 3 times in phosphate buffered saline (PBS), were extracted in a similar manner. Lipid extracts were checked for radioactivity by spotting 50  $\mu$ l of extract on a piece of filter paper. This paper was then air dried, cut into small pieces, placed in a scintillation viral and counted for  $P^{32}$  activity in a Packard 3375 Tri-carb scintillation spectrophotometer. Labeled lipid extracts containing 50,000 to 80,000 counts per minute (cpm) were analyzed for phospholipid composition by preparing deacylated derivatives by mild alkaline and acid hydrolysis according to the method of Wells and Dittmer (3). These derivatives were chromatographed in two dimensions on large sheets of Whatman No. 4 paper in phenol:water (100:38) and butanol: propionic acid:water (142:71:100) solvents, according to the procedure of Benson and Maruo (4). In addition, compounds such as sphingomyelin, that were not affected by the mild acid or alkaline hydrolysis, were chromatographed by thin-layer chromatography on Silica gel G plates in a chloroform:methanol:water (65:25:4) solvent system. Unknown phosphatides were tentatively identified by comparison with known standards or by comparing the Rf values of the deacylated products with those reported by Benson and Strickland (5). In both paper and thin-layer chromatography, radioautography was used to locate the isotopically-labeled compounds. Radioactive areas on thin-layer plates and chromatograms of the deacylated derivatives corresponding to the spots on the radioautograms were removed and quantitated by scintillation spectrophotometry.

Gas chromatography was initiated with fatty acid methyl esters (FAME) produced by acid methanolysis of the phospholipid samples. Phospholipid fractions for gas chromatography were prepared by removing the neutral lipids through the use of thin-layer chromatography. Lipid samples spotted across the bottom of an 8 X 8 inch glass plate coated with Silica gel G were chromatographed in a hexane:chloroform:acetic acid:methanol (120:20:2:1) solvent system. The phospholipid fraction, which remained at the origin, was removed from the Silica gel by twice extracting with chloroform:methanol (1:2). These phospholipid fractions of virus and cell samples were evaporated to dryness under nitrogen, hydrolyzed with 0.5 per cent sulfuric acid in methanol at 75 C for one hour, and extracted for FAME with hexane. Methyl esters were chromatographed in an F and M model 810 gas chromatograph equipped with a dual flame

420

ionization detector. The columns were 8-foot X 4-mm copper, packed with 6 per cent LAC-3R-728 (DEGS) on 100/120 mesh gas chrom Q. The column was operated isothermally at 180 C and resulting peaks were compared with relative retention times of a series of known standards.

#### RESULTS AND DISCUSSION

Biological Characterization of VEE Virus. Previous results have shown that the chick embryo host maintained the large-plaque characteristic and virulence properties of the virus during passage, but passage of the virus in CF cells rapidly produced populations that were intermediate with respect to plaque size and virulence (1). Experiments with VEE virus which had been altered by 10 passages in CF cells and then injected into embryonated eggs were initiated to give additional information on the biological characteristics as well as lipid content. A scheme of the virus passages and summary of the biological characteristics is shown in Table 1. Note that upon inoculation of the virus from the 10th passage in CF cells into embryo, an immediate reversal of the intermediate plaque size and virulence trend is seen, in that large plaques are found in the resulting virus population, and an increase in ip virulence for mice is found. The results of these experiments indicate that the host is highly instrumental in the selection of VEE virus types possessing different plaque-forming capabilities and different levels of virulence. Evidence that as the large-plaque characteristic was lost the virus showed an increased degree of attenuation for test animals, and conversely when the large-plaque characteristic was regained a decrease in the degree of attenuation supports the hypothesis that these two properties are closely associated during passage in cell culture.

The role virus lipids play in the infective process other than a mechanical or passive function is not clear. Speculation has been made that the virus lipids which are derived from preformed host cell lipids have a special function such as involvement in the adsorption of the virus to the host cell membrane. To examine the role played by the lipids of VEE virus in the virus selection process during passage in cell culture, a comprehensive analysis of the phospholipid and fatty acid fractions from purified virus preparations and host cell tissue was undertaken.

Host Cell and Virus Phospholipid. To compare the phospholipid fraction of the embryo-derived virus with that of the CF-derived virus,  $P^{32}$ -labeled lipid fractions from host cells and purified virus preparations were subjected to mild alkaline and acid hydrolysis to produce deacylated derivatives which were separated by paper chromatography. A typical example of a radioautogram of the deacylated phospholipids is shown in Figure 1. The percentage composition of the deacylated glycerophosphate (GP) esters of uninfected and infected embryo and of purified embryo-derived VEE

TABLE 1  
HOST INFLUENCE ON PLAQUE FORMATION AND MOUSE VIRULENCE BY VENEZUELAN  
EQUINE ENCEPHALOMYELITIS VIRUS

Host	Inoculum	Progeny Virus	Plaque Size <sup>a/</sup>			Mouse Virulence Titer <sup>b/</sup> IC <sub>50</sub> / IP <sub>d</sub> / IC-IP <sub>e</sub>
			L	I	S	
Chick Embryo	Embryo	Embryo Parent	100 <sup>f/</sup>	0	0	9.5
	Egg Seed	Egg Strain				9.0
CF Cell	Embryo	CF Cell - 1st Passage (CF1P)	100	0	0	8.8
	Egg Seed					8.5
CF Cell	CF cell	CF Cell - 10th Passage (CF10P)	0	87	13	9.5
	9th Passage					7.5
Chick Embryo	CF cell	Embryo	13	87	0	9.5
	10th Passage	CF Cell Strain				8.4
						1.1

a. Large 4-6 mm; intermediate 2-3.5 mm; small 0.5-1.5 mm.

b. Log<sub>10</sub> LD<sub>50</sub>/ml.

c. Titration in mice by the intracerebral route.

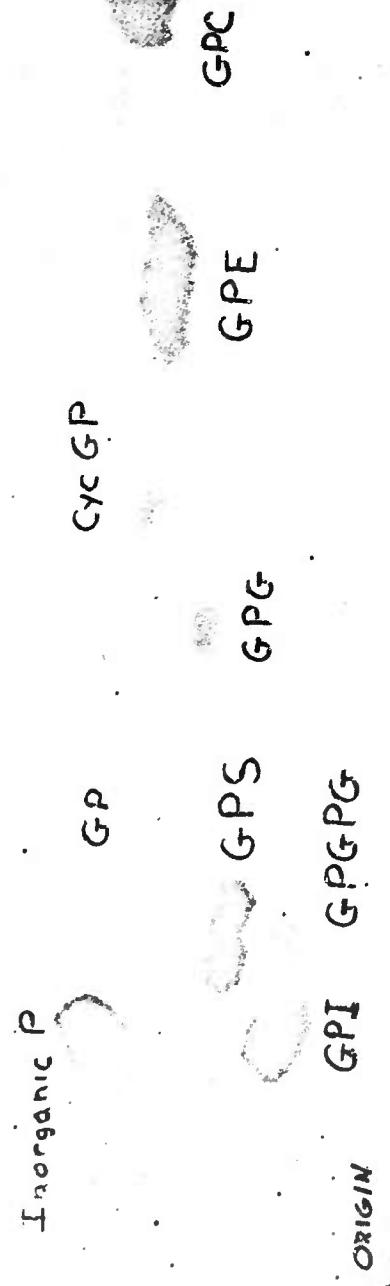
d. Titration in mice by the intraperitoneal route.

e. Difference between titers obtained by intracerebral and intraperitoneal route.

f. Incidence of large plaques ranged from 95 to 100 per cent of total. Other results are also expressed as per cent of total.

HEYDRICK, WACHTER and DWYER

Butanol : Propionic acid : water (142:71:100)



Phenol : water (100 : 38)

Figure 1. Radioautogram of deacylated phospholipids. Abbreviations used: prefix GP, glycerophosphoroxy; I, inositol; S, serine; G, glycerol; E, ethanolamine; C, choline. Abbreviations of other compounds: GP, glycerol phosphate; Cyc GP, cyclic glycerophosphate; GPGC, diglycerol phosphoroglycerol.

423

HEYDRICK, WACHTER and DWYER

virus is shown in Table 2. A total of 10 glycerophosphate esters were present in both the host and virus, the most predominant being GP-choline. Infection of the embryo host by the parent egg seed VEE virus caused a reduction in the percentage of most all phospholipid components except GP-choline which increased in concentration. Purified VEE virus derived from the embryo host did not reflect the change in the host phospholipid fraction brought about by infection, but had a composition that quantitatively differed from the normal and infected host phospholipids. In addition to a reduced value for GP-choline and an increased amount of GP-serine and GP-ethanolamine (phosphatidyl ethanolamine plus ethanolamine plasmalogen), an unidentified glycerophosphate ester was found to be present in much larger quantities in the virus than in the host. This component was characterized as having an Rf of 0.04 in the phenol-water phase and an Rf of 0.05 in the butanol-propionic acid-water phase.

Infection of CF cells with the large-plaque VEE virus of embryo origin produced a less marked change in the glycerophosphate ester distribution in the host cells than did infection of the chick embryo host (Table 3). In addition to a smaller increase in GP-choline, a significant increase in GP-inositol was found in infected CF cells, whereas the same component was decreased by infecting the embryo host. As in the embryo-derived virus preparations, the CF cell-derived virus showed quantitative differences in phospholipid composition when compared to the host cell phospholipid composition. When the phospholipid fraction extracted from large-plaque embryo-derived virus was compared to the phospholipid fraction of large-plaque CF cell-derived virus it was found that a markedly similar pattern of relative percentages existed for the same glycerophosphate esters (Tables 2 and 3). From these results it would appear that although the virus was passed from one type of host to another, the virus phospholipid composition was not altered. Possibly the close genetic relationship of the chick embryo and CF cell host might account for this similarity, i.e., the virus may obtain these lipids from a source which is similar in both hosts.

As indicated previously, the CF cell host selects virus populations which are intermediate with respect to plaque size and virulence. In order to determine the role of the virus lipids in relation to these changes in biological characteristics the phospholipid fraction from VEE which had been passed 10 times in the CF cell host was analyzed. A comparison of the glycerophosphate esters of cells infected with the intermediate plaque size (CF10P) VEE virus with the same components of cells infected with the large plaque (CF1P) VEE virus indicated a similarity in percentage composition of individual glycerophosphate esters (Tables 3 and 4). In an identical comparison of the phospholipid composition of the intermediate plaque virus with the phospholipid composition of the large plaque virus, a marked resemblance in the phosphatidic percentage composition of the two viruses was also found.

HEYDRICK, WACHTER and DWYER

TABLE 2

THE GLYCEROPHOSPHATE ESTER COMPOSITION OF UNINFECTED  
AND INFECTED CHICK EMBRYO AND OF VEE VIRUS PURIFIED  
FROM CHICK EMBRYO

Component	Per Cent Composition		
	Uninfected Embryo	Infected Embryo	PES <sup>a</sup> / Virus
Unknown	0.19	0.08	1.58
GP Inositol <sup>b</sup> /	12.79	9.01	4.75
GPGP Glycerol	2.59	1.37	0.38
GP Serine	7.05	4.73	12.90
Glycerol phosphate	2.33	1.17	2.39
GP Glycerol	1.41	1.76	0.69
GP Ethanolamine	11.98	9.22	9.85
GPE-Plasmalogen	4.30	2.75	11.48
GP Choline	51.64	65.51	48.43
Sphingomyelin	3.41	3.00	7.55

a. Parent egg strain.

b. Prefix GP = glycerophosphoro.

425

HEYDRICK, WACHTER and DWYER

TABLE 3

THE GLYCEROPHOSPHATE ESTER COMPOSITION OF UNINFECTED AND INFECTED CHICK FIBROBLAST CELLS AND OF VEE VIRUS PURIFIED FROM CHICK FIBROBLAST CELLS

Component	Per Cent Composition		
	Uninfected CF Cells	Infected CF Cells (CF1P)	CF1P Virus
Unknown	0.01	0.08	1.02
GP Inositol <sup>b/</sup>	5.33	8.36	2.58
GP GP Glycerol	1.97	2.35	0.70
GP Serine	8.84	7.39	16.96
Glycerol phosphate	2.05	1.29	2.57
GP Glycerol	4.76	4.61	0.85
GP Ethanolamine	12.13	9.21	8.33
GPE-Plasmalogen	10.08	8.32	12.94
GP Choline	44.30	52.77	41.76
Sphingomyelin	9.81	4.75	12.50
Cyclic glycerol phosphate	0.50	1.06	0.74

a. Chick fibroblast 1st passage.

b. Prefix GP = glycerophosphoro-

426

TABLE 4

THE GLYCEROPHOSPHATE ESTER COMPOSITION OF UNINFECTED  
AND INFECTED CHICK FIBROBLAST CELLS AND OF VEE VIRUS  
PURIFIED FROM CHICK FIBROBLAST CELLS

Component	Per Cent Composition		
	Uninfected CF Cells	Infected CF Cells (CF10P)	CF10P <sup>a</sup> / Virus
Unknown	0.05	0.08	0.60
GP Inositol <sup>b</sup> /	5.33	7.54	1.69
GPGP Glycerol	1.97	2.47	0.19
GP Serine	8.84	6.60	17.56
Glycerol phosphate	2.05	1.52	3.34
GP Glycerol	4.16	3.51	0.80
GP Ethanolamine	12.13	11.02	6.83
GPE-Plasmalogen	10.68	9.02	16.04
GP Choline	44.30	51.20	41.00
Sphingomyelin	9.81	6.31	10.19
Cyclic glycerol phosphate	1.00	1.50	0.40

a. Chick fibroblast 10th passage.

b. Prefix GP = glycerophosphoro-

427

HEYDRICK, WACHTER and DWYER

From the results obtained in the analysis of the phospholipid fractions from various VEE virus types, it can be concluded that the glycerophosphate esters of phospholipid associated with the virus surface structure, i.e., envelope, do not appear to be a major factor in influencing the selection by the host of the virus plaque size or the related virulence characteristic, since the glycerophosphate ester character of the virus did not change through 10 passages in CF cells.

Fatty Acids of the Host Cell and Virus Phospholipids. The number of fatty acids present in the VEE virus and host cell phospholipid samples ranged from approximately 25 for virus preparations to about 30 for the cell phospholipids. Palmitic (C16), stearic (C18), and oleic (C18:1) were by far the more predominant acids, comprising approximately 55 per cent of both the host cell and virus phospholipid fatty acids.

The data represented in Table 5 represents an excellent example of the host influence on the fatty acid content of the virus particle. In the embryo host phospholipids, the major fatty acid was palmitic acid (C16). This fatty acid was also the major acid in the embryo-propagated virus phospholipid. When embryo-derived virus of the palmitic acid (C16) type was inoculated into the chick fibroblast host, the resulting chick fibroblast-derived progeny (CF1P) possessed a phospholipid fatty acid composition in which stearic (C18) was the predominant acid. Stearic acid (C18) was also the predominant fatty acid of the host CF cell phospholipid fraction. The predominance of stearic acid (C18) as the major fatty acid in both host cell and virus phospholipid fractions was maintained through 10 passages. However, when the stearic acid (C18)-type, 10th passage virus preparation was used as inoculum for embryonated eggs, the resulting embryo-derived virus (embryo-virus-CF cell strain) possessed a phospholipid fatty acid composition in which palmitic acid (C16) was again the predominant acid.

Whether the alteration of the phospholipid fatty acids from palmitic (C16) to stearic (C18) acids by the CF cell host is a prerequisite for the selection of intermediate plaque size virus populations is not known. The failure to detect any other alteration in the virus phospholipid fatty acid content during passage would suggest that this is probably not the case.

#### SUMMARY AND CONCLUSION

The goal of the present study was to establish a comprehensive analysis of VEE virus and host cells from various passage levels in terms of fatty acids and phospholipids, and to correlate any changes in the lipid composition with host-induced changes in the biological activity of the virus during passage.

TABLE 3

PERCENTAGE COMPOSITIONS OF THE PHOSPHOLIPIDS RECOVERED  
FROM VARIOUS VESSELS AND FROM PURIFIED VEE VIRUS

Phospholipid Sample	Palmitic (C16)	Stearic (C18)	Oleic (C18:1)
Embryo - Parent Egg Strain	27.11	17.14	13.79
Infected Embryo	23.57	10.40	12.14
Virus			
CF Cell - First Passage			
Infected CF Cell	15.49	23.90	17.70
Virus	19.06	21.42	15.76
CF Cell - 10th Passage			
Infected CF Cell	14.47	23.71	21.37
Virus	18.23	23.23	14.58
Embryo - CF Cell Strain			
Infected Embryo	25.61	19.18	17.06
Virus	25.08	13.34	15.32

46  
82  
9

BUCHICK, MACKENZIE AND DWYER

Although VEE virus is altered in plaque size and virulence following passage in CF cell monolayers, no corresponding change was noted in the phospholipid composition of the virus. The data indicated however, that the virus phospholipid fatty acids did change after only one passage in CF cells. The predominant phospholipid fatty acid associated with virus derived from the chick embryo host was palmitic acid (C16). It was shown previously that virus passage in the chick embryo host did not alter plaque size or virulence. When virus of chick embryo origin was passed one time in CF cells, a virus population was produced where stearic acid (C18) replaced palmitic acid (C16) as the predominant phospholipid fatty acid. This composition was maintained for 9 additional passages. The significance of host influence on the phospholipid fatty acids in the selection of intermediate plaque size virus populations is not known.

The expression of phenotypic, surface-associated properties by lipid-containing viruses appears to be influenced by a complex arrangement of several factors such as the type of host cell, the chemical and physical makeup of the lipoprotein cell membrane, the genetic makeup of the infecting virus, and the area of virus saturation in the cell. With these factors in mind, and taking into consideration the information gained through the analysis of the host cell and VEE viral phospholipids in this study, it should prove very useful in future approaches to examine the role of the host cell in the control of phenotypic virus properties which might provide virus populations with increased resistance to a variety of incanting stresses.

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